Techniques to improve neurological outcome after cardiac surgery

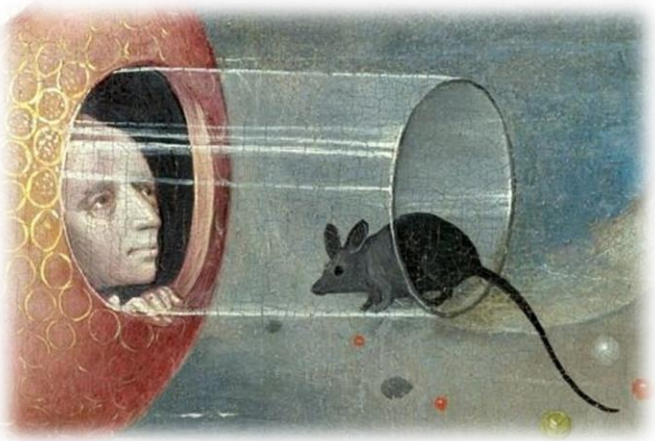
Kok, Linde

Chapter 8

Mild and deep hypothermia differentially affect cerebral neuroinflammatory and cold shock response following cardiopulmonary bypass in rat

W.F. Kok, A.H. Epema, M. Houwertjes, H. Mungroop, J. Doorduin, T.W.L. Scheeren, H.R. Bouma, R.H. Henning, A.R. Absalom

Manuscript in preparation



Abstract

Introduction: Neuroinflammation is considered to play a role in the pathogenesis of neurocognitive complications after cardiac surgery, particularly among elderly patients and those undergoing cardiopulmonary bypass (CPB) assisted procedures. Targeted temperature management (TTM) serves is a potentially neuroprotective strategy during cardiac surgery, possibly through activation of cold shock proteins, but data on its effectiveness in attenuating neuroinflammation are lacking. Therefore, we investigated the effects of mild compared to deep hypothermia on the neuroinflammatory response and cold shock protein expression after CPB in rats.

Methods: Adult male Wistar rats were subjected to 1 hr of TTM at 18^{o}C or 33^{o}C during a CPB or sham procedure under ketamine anesthesia. Arterial blood samples were obtained at 15 and 55 minutes after start of cooling and during weaning. Neuroinflammation was investigated by serial positron emission tomography (PET) scans of uptake of TSPO-binding ligand [^{11}C]-PBR28, prior to and at day 1, 3 and 7 after the procedure. Hippocampal and cortical regions were obtained at day 1 or day 7. Cold shock protein RNA-binding motive 3 (RBM3) and tyrosine receptor kinase B (TrkB) receptor expression were quantified with Western Blot analysis.

Results: TTM was applied with similar cooling and rewarming rates in CPB and sham groups, allowing all rats to reach target temperature within the hour. Standard uptake values (SUV) of [11C]-PBR28 in CPB rats at 1 day and 3 days were similar to that in Sham animals. However, at 7 days after CPB, SUV was significantly increased in amygdala and hippocampal regions of rats from the CPB 18^{o}C group compared to the CPB 33^{o}C group. Additionally, RBM3 protein levels in cortex and hippocampus were significantly higher in CPB 33^{o}C compared to CPB 18^{o}C and sham 33^{o}C at day 1 and day 7, respectively.

Conclusions: Deep hypothermia was associated with an increased neuroinflammatory response in amygdala and hippocampus compared to mild hypothermia in rats that underwent a cardiopulmonary bypass procedure. Additionally, mild hypothermia was associated with increased expression of TrkB and RBM3 in cortex and hippocampus of rats on CPB compared to deep hypothermia. Together, these data indicate that neuroinflammation is attenuated

by mild hypothermia, possibly by recruiting protective mechanisms through cold shock protein induction.

Introduction

Neurocognitive dysfunction is a common complication after cardiac surgery, affecting 50-70% of patients within the first postoperative week and 20–40% after 6 months to a year (Newman et al., 2006). Although debated (Kennedy et al., 2013), we found the use of cardiopulmonary bypass to be an independent risk factor for the development neurocognitive dysfunction (Kok et al., 2014). Despite substantial improvements in cardiopulmonary bypass technology, and surgical and anesthetic techniques, neurological injury remains a concern in the increasingly elderly patient population undergoing cardiac surgery. In a response to ischemia-reperfusion (I/R) injury, the innate immune system serves to minimalize neuronal injury, initiated by an activation and migration of microglial cells (Feuerstein et al., 1998). Targeted temperature management (TTM) is a well-known strategy against neurological injury induced during and after cardiac surgery (Antonic et al., 2014). TTM research focuses mainly on establishing the ideal target temperature to prevent neurological outcome. According to our recent findings in a retrospective study of a large cohort of patients who underwent cardiopulmonary bypass-assisted cardiac surgery, mild hypothermia (33^{o}C) is the target temperature that optimally enhances postoperative survival (Kok et al., Chapter 7). Currently, there is no evidence relating TTM targets to neuroinflammation associated with CPB. Hence, it would be worthwhile to investigate neuroinflammation at mild and deep hypothermia.

We recently developed a rat CPB model that avoids the need for blood transfusion (Samarska et al., 2013), and found CPB to induce substantial systemic and renal inflammation (Bouma et al., 2013). Therefore, we consider the model highly suitable for comparison of the neuroinflammatory response after CPB under mild hypothermic (33^{o}C) and deep hypothermic (18^{o}C) conditions. Microglial activation can be monitored *in vivo* with PET-scans after administration of [^{11}C]-PBR-28, a second-generation Translocator protein (TSPO) binding ligand. In comparison with older and more widely used [^{11}C]-

PK11195, [^{11}C]-PBR-28 has a higher sensitivity to detect microglial activation (Parente et al., 2016). As the tracer has a short half-life, the scans can be repeated allowing serial assessment of microglial activation.

Hypothermia can induce cold shock proteins, such as RNA binding motive 3 (RBM3), known for its capacity to regenerate synapses and improve neurological function (Peretti et al., 2015). RBM3 is suggested to be upregulated through activation of tyrosine receptor kinase B (TrkB) receptor, which constitutes an important regulator of neurotrophins, such as BDNF (Ascaño et al., 2009). Thus, we examined levels of RBM3 and TrkB receptor, as its expression levels might change at mild and deep hypothermia. The aim of this study is to investigate the influence of CPB on neuroinflammation, by comparing the neuroinflammatory response in relation to the expression of cold shock proteins at mild and deep hypothermia.

Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen and guidelines for standards of conduct of animal studies were followed. Adult male Wistar Crl rats from Charles River, Germany, with a body weight >450 gram were included in the experiment. Randomization was performed to target temperature management at 18^{o}C or 33^{o}C during CPB or sham and short-term (at day 1 post-procedure) or long-term (at day 3 and day 7 post-procedure) PET-scan regimen (8 groups, n=3-4 per group). As from prior to the first PET-scan, the rats were individually housed. Animals were kept at a 12hr light/dark cycle, with free access to food and water.

Anesthetic procedure

Anesthesia was induced with 5% isoflurane and maintained through a nose cone at 2-3% in O_2/air (1:1). Rats underwent tracheal intubation and mechanical ventilation at a respiratory rate of 80 min^{-1}. The tail vein was cannulated to enable intravenous anesthetic agent administration. Atropine (40 µg.kg^{-1}) was

administered s.c. as a single dose to prevent excessive airway secretions. Temperature was continuously monitored with a flexible rectal probe. Rocuronium (0.5 $mg.kg^{-1}$) was used as a muscle relaxant in repeated bolus doses. The left femoral artery was cannulated for blood pressure monitoring. Anesthesia was then switched to continuous intravenous ketamine (70 $mg.ml^{-1}$) adjusted manually to maintain the mean arterial pressure between 60 and 100 mmHg by adjusting the infusion speed between 1.2 and 1.5 ml/hr. Fentanyl (40 $\mu g.kg^{-1}$) divided in small doses was administered i.v. during CPB.

CPB procedure

The experimental protocol for the CPB was modified from the model developed for extracorporeal circulation in the rat by Samarska et al. (2013), featuring a miniaturization of the circuit allowing CPB without the need for blood transfusion and additional hypothermia induction through cooling of the CPB circuit. Before insertion of the cannulae for CPB, 3000 $IU.kg^{-1}$ heparin was administered. To enable CPB, the left carotid artery was cannulated for arterial inflow using a 22-gauge catheter. A multi-orifice 4.5 French cannula was advanced into the right atrium through the right jugular vein.

The extracorporeal circulation setup consisted of a glass venous reservoir, a peristaltic pump (Watson Marlow 120S), a neonatal membrane oxygenator and a glass counter-flow heat exchanger with built-in bubble trap. All components were connected with polyethylene tubing (1.6 mm inner diameter). The venous reservoir and heat exchanger were cleaned and sterilized prior to use. The CPB circuit was primed with 12 ml of heparinized (90 IU) Haes 6.0% solution.

During the procedure, pulse and blood oxygen saturation are continuously monitored (MouseOxPlus pulse oximetry, Harvard apparatus). Targeted CPB flow was 100-120 $ml.kg^{-1}.min^{-1}$. After starting CPB, anesthesia was reduced to approximately 0.6 ml/hr in 33^{o}C temperature groups and to 0.2 ml/hr in 18^{o}C temperature groups. Blood gas analysis was performed by α-stat method, on 100µl blood samples taken from the cannula in the left femoral artery at 15 and 55 min after start of CPB, and after removal of the CPB cannulae in the postoperative phase. Animals were on the CPB machine for 1 hour, after which rewarming was initiated. Body temperature (Tb) was adjusted during CPB by cooling with ice packs or heating of the blood circuitry in addition to an external

cooling/heating pad. After weaning from CPB and disconnection of the circuit, a single dose of protamine (1500 $IU.kg^{-1}$ i.v.) was administered. After removal of the cannulae, the wounds were sutured and anesthesia was weaned. The tracheal tube was removed when the animal regained consciousness. Buprenorphine (10 $\mu g.kg^{-1}$) was given once s.c. as a postoperative analgesic. The experiments were performed between 8:00 and 12:30, except for the PET-scans which were performed between 15:00 and 17:30.

PET scans

In order to evaluate the neuroinflammatory response, PET scans were performed with TSPO binding [11C]-PBR28 tracer. As the scans were performed in vivo, we were able to follow-up the neuroinflammatory process prior to CPB and at day 1, 3 and 7 after the CPB procedure. After transfer to the small-animal PET facility, anaesthesia was induced in non-fasted rats and maintained with isoflurane 2%. The [^{11}C]-PBR28 tracer (12.1±3.7 MBq, 7.6 ± 0.5 $ng.l^{-1}$), diluted in saline 0.9% was administered via the penile vein. After injection, anaesthetic administration was stopped and rats were awake for 45 minutes. Thereafter they were again anesthesized with isoflurane, to be positioned in the µPET camera. After a transmission scan with [^{57}C], the animals underwent a scan of 30 minutes. The list-mode data of the emission scans was compressed into a single time frame (45-75 minutes after injection). Emission sinograms were iteratively reconstructed (OSEM2D). After recovery in a quiet environment, animals were transferred back to the animal facility.

After the last PET-scan, the animals were sacrificed under deep anesthesia by exsanguination through the abdominal aorta, and flushed with saline for 2 minutes. Brain tissue was dissected according to brain regions, including amongst others cortex and hippocampus.

PET image analysis

PET scan data were analyzed using PMOD 3.6 software (PMOD Technologies Ltd). The scans were automatically registered to [^{11}C]-PBR28-specific PET templates, constructed similar to the method described by Vállez Garcia et al. (2015). Volumes of interest (VOIs) of several brain regions were constructed on the basis of previously defined structures (Vállez Garcia et al., 2015). The brain

radioactivity concentration was calculated from the VOIs and expressed as standard uptake values (SUV): averaged tissue activity concentration [kBq/cc] / (injected dose [MBq] / body weight [g]).

Western blot

Brain samples were homogenized in a RIPA-TBS buffer followed by centrifugation for 20 min at 14,000 rpm at 4^{o}C. The quantity of protein was determined in a Bradford protein assay (BioRad, The Netherlands). Equal amounts of total protein in SDS-PAGE sample buffer were separated on SDS-PAGE 4-20% PreciseTM Protein gels (Thermo Scientific). After transfer to nitrocellulose membranes (BioRad), proteins were blocked in 5% bovine serum albumin. Membranes were then incubated overnight at 4^{o}C with primary antibodies, followed by incubation with secondary antibodies in 1h at room temperature. The following primary antibodies were used: rabbit polyclonal anti-RBM3 (14363-1-AP, Proteintech, UK) and rabbit polyclonal anti-TrkB (4606S, Cell Signaling Technology, USA), with rabbit monoclonal β-actin (4967S, Cell Signaling Technology, USA) as loading control. Secondary antibody used was horseradish peroxidase-conjugated anti-rabbit (W4018, Promega, USA). Signals were detected by Novex ECL HRP Chemiluminescent Substrate reagent kit (WP20005, Invitrogen, USA) and quantified by densitometry with software from ChemiDoc (BioRad, UK).

Statistical analysis

Statistical calculations were performed using SPSS 22.0 for Windows. Sigmaplot 13.0 was used for creating figures. Normality of distribution was tested by Kolmogorov-Smirnov tests. Differences in continuous data were examined using ANOVA for parametrical and with Kruskal Wallis for non-parametrical data. Data are presented as mean ± SEM, or median (25^{th}-75^{th} percentile). Differences were considered significant at $p<0.05$.

Results

Pre-operative weights and weight losses

A total of 39 rats were randomized for procedures, of which 30 completed the procedure and PET-scans. Nine animals were excluded from the study, mainly related to pulmonary problems following weaning of CPB at 18°C (n=4) and technical problems while the animal was on CPB (n=2). Additionally, one animal died of hypotensive shock directly after weaning of the CPB at 18°C and 2 animals were found dead in their cages within 24h after CPB at 33°C.

Cooling rate

All animals were cooled according to a standardized protocol, utilizing a cooling mattress underneath the animal and by internal application of hypothermia with cooled blood from the CPB circuit or by external application of ice packings to the abdomen in sham operated rats (Dugbartey et al., 2015). Despite deep hypothermia in sham animals being solely dependent on external cooling, we were able to cool them at a similar rate as CPB rats (Figure 1), reaching the targeted temperature of 33°C in 10 (8-16) min in CPB and 7 (6-7) min in sham group. Cooling to 18°C was accomplished in 48 (45-49) min in CPB and 54 (52-56) min in sham group.

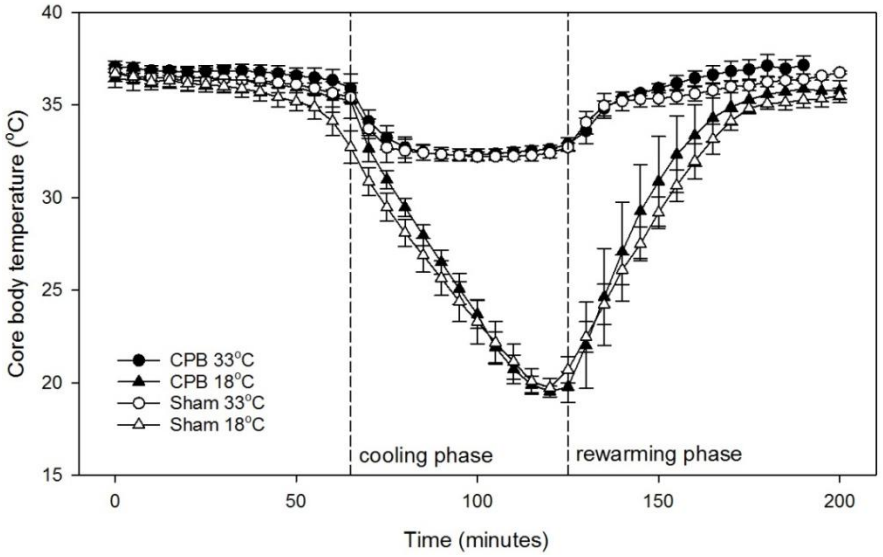


Figure 1. Core body temperature registration of CPB and sham rats at 18°C and 33°C.

Arterial blood gas analysis

Blood samples were taken at 15 min after initiation of cooling (Tb = 30-33^{o}C), after 55 minutes of cooling (Tb=33^{o}C or 18^{o}C), and when the cannulae were removed during weaning (Tb=35^{o}C). Arterial blood gases were analyzed using α-stat method (Figure 2). As a consequence, pH values of blood were relative high in all groups; after 15 minutes cooling the mean pH amounted 7.5 in all groups. Thereafter, pH values remained above 7.45 without significant differences between CPB and sham. Base excess (BE) was significantly lower at 15 minutes of cooling in CPB 18^{o}C rats compared to its sham, whereas it was significantly higher during weaning in CPB 33^{o}C operated rats compared to its sham. pCO_2 levels were all relatively low, and differed only during weaning, with pCO_2 in CPB 18^{o}C being significantly higher than in its sham group. From the start of CPB, pO_2 decreased significantly in both the CPB 18^{o}C and CPB 33^{o}C groups. After 55 minutes of cooling at 33^{o}C, rats still showed lower pO_2 compared to sham, whereas in deep hypothermia pO_2 in CPB rats did not differ from sham. Additionally, arterial saturation showed a similar pattern of decreased values at 15 min of cooling in both CPB temperature groups, whereas it remained normal in both ventilated sham groups. Hematocrit values were significantly decreased in all CPB operated rats, due to the volume priming of the circuit. However, differences between CPB and sham rats disappeared after i.v. administration of whole blood that was collected from the CPB system during weaning from anesthesia. Blood glucose and lactate levels were significantly higher in both CPB temperature groups at 55 minutes after initiation of cooling, yet normalized upon weaning. Thus, apart from significant hemodynamic changes that occur during cooling on CPB, most variables return to control values during weaning. In addition, blood gas data of rats at 33^{o}C CPB appeared to be less advantageous when compared to 18^{o}C CPB, e.g. higher lactate levels at 15 min of cooling, and decreased sO_2 and pO_2 at 55 min of cooling.

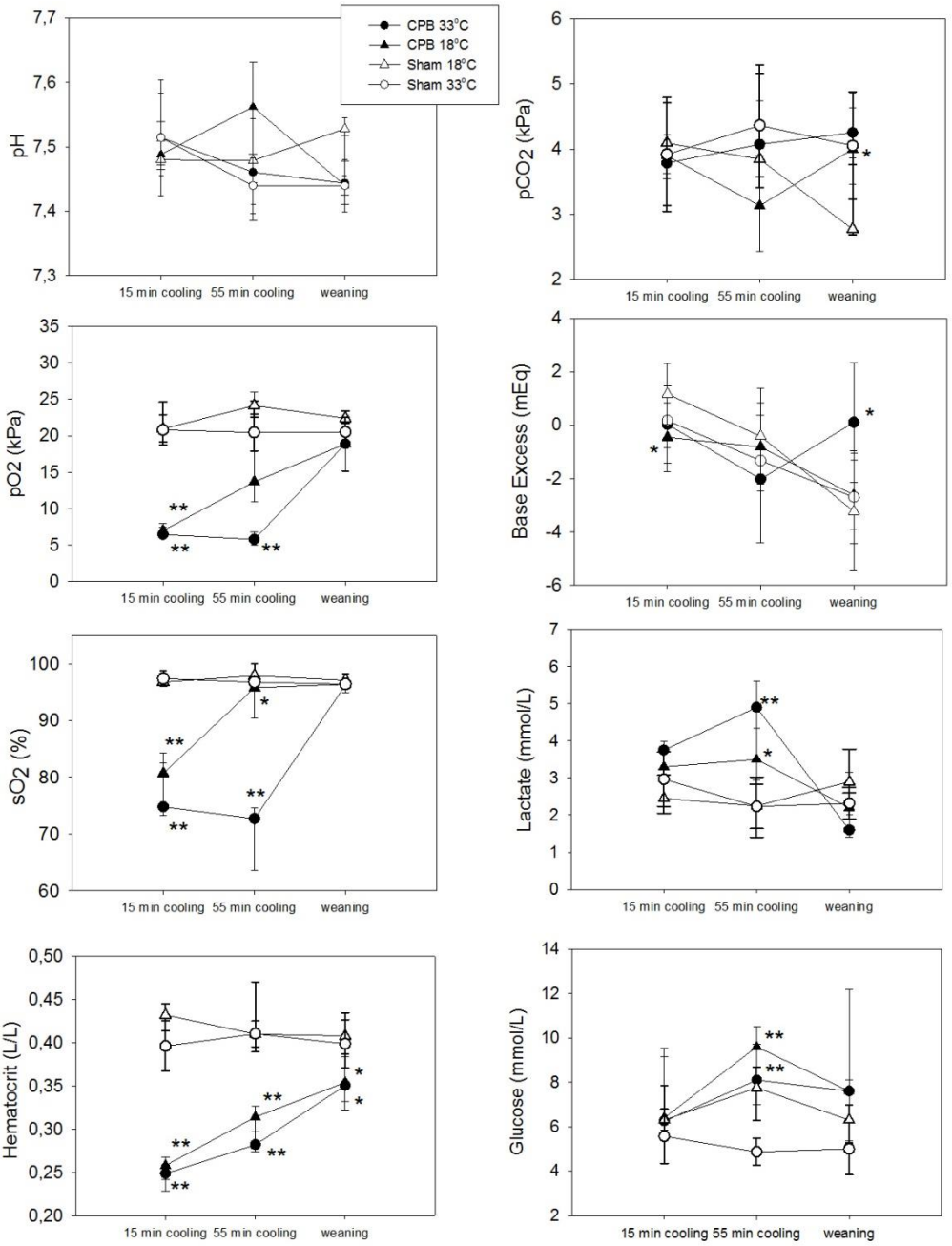


Figure 2. Arterial blood gas measurements during CPB or cooling in sham procedure, taken at 15 minutes or 55 minutes of after start cooling and during weaning. Significant differences are indicated with */** for $p<0.05$ and $p<0.01$ respectively, compared to its sham group.

PET analysis

Axial sliced PET images of [^{11}C]-PBR28 in CPB and sham operated rats at 18^{o}C and 33^{o}C at day 1, day 3, and day 7 after procedures are presented in Figure 3. They reveal a high tracer uptake in 18^{o}C CPB group, seen as a hot-spot of fluorescent green in the left anterior hemisphere. Overlay with the PET template revealed the area to be the anterior part of hippocampus. Cerebral uptake of [^{11}C]-PBR28 tracer per region of all rats after undergoing CPB procedure are presented in Table 1. Pre-operative SUV in all brain regions were the same for all experimental groups, and similar to the SUV at 1 day after procedure (Suppl. 1). At 1 day after CPB, there is no marked difference between SUV in any brain region for 18^{o}C compared to 33^{o}C. After 3 days, SUV were similar between temperature groups, with a tendency of higher values in the 18^{o}C group (Figure 4). Subsequently, SUV in hippocampal and amygdala regions increased significantly in the 18^{o}C group at 7 days after CPB compared to rats who underwent CPB at 33^{o}C ($p=0.031$ and $p=0.025$, respectively; Figure 4). In all sham operated rats, there were no significant differences in SUV between temperature groups or compared to baseline at any time point, nor was there a tendency of higher SUV in 18^{o}C compared to 33^{o}C (Suppl. 2).

To follow-up SUV throughout time, we correlated pre-procedure scan SUV of all brain regions with SUV obtained day 1, 3 and 7 after procedures. There were no correlations between pre-procedure scan data and data obtained at any time point after CPB and sham. However, when analyzing SUV obtained at day 3 and day 7, originating from the same animals, we found highly significant correlations in amygdala ($r=0.946$), hippocampus ($r=0.907$), globus pallidus ($r=0.964$), striatum ($r=0.927$), and cortex ($r=0.935$), with $p<0.001$ in all brain regions, indicating an ongoing neuroinflammatory response. In contrast, no correlations between day 3 and day 7 levels were found in brainstem, cerebellum, pre-frontal cortex and hypothalamus.

Table 1. Cerebral uptake per brain region of [^{11}C]-PBR28, expressed as standardized uptake values (SUV; median (25^{th} – 75^{th} percentiles)), as determined in rats on cardiopulmonary bypass at 18°C and 33°C at 1 day, 3 days and 7 days after procedures. * denotes a significant difference of $p<0.05$ between temperature groups.

Brain area	1 day after CPB		3 days after CPB		7 days after CPB	
	18°C	33°C	18°C	33°C	18°C	33°C
Amygdala	**0.28 (0.27-0.37)**	**0.29 (0.29 -0.35)**	**0.46 (0.31-1.16)**	**0.31 (0.27-0.51)**	**0.59 * (0.45-0.95)**	**0.29 (0.28-0.30)**
Cerebellum	**0.37 (0.29-0.63)**	**0.46 (0.40-0.52)**	**0.54 (0.39-0.55)**	**0.44 (0.37-0.50)**	**0.56 (0.49-0.58)**	**0.40 (0.32-0.49)**
Globus Pallidus	**0.17 (0.16-0.30)**	**0.19 (0.16-0.27)**	**0.44 (0.31-1.07)**	**0.29 (0.23-0.34)**	**0.74 (0.46-1.02)**	**0.20 (0.17-0.30)**
Thalamus	**0.44 (0.26-0.51**	**0.37 (0.30-0.41)**	**0.60 (0.42-0.80)**	**0.40 (0.30-0.43)**	**0.74 (0.56-0.83)**	**0.36 (0.32-0.44)**
Hippocampus	**0.31 (0.22-0.35)**	**0.32 (0.28-0.36)**	**0.44 (0.35-0.98)**	**0.29 (0.27-0.46)**	**0.53 * (0.40-0.84)**	**0.32 (0.29-0.35)**
Striatum	**0.24 (0.22-0.30)**	**0.27 (0.23-0.33)**	**0.47 (0.38-0.96)**	**0.29 (0.26-0.48)**	**0.57 (0.41-0.83)**	**0.30 (0.24-0.39)**
Brainstem	**0.33 (0.27-0.43)**	**0.43 (0.37-0.47)**	**0.45 (0.31-0.47)**	**0.42 (0.30-0.60)**	**0.52 (0.46-0.52)**	**0.43 (0.36-0.50)**
Prefrontal Cortex	**0.50 (0.34-0.71)**	**0.58 (0.44-0.66)**	**0.75 (0.63-0.81)**	**0.53 (0.38-0.77)**	**0.66 (0.62-0.74)**	**0.63 (0.52-0.73)**
Cortex	**0.42 (0.29-0.57)**	**0.44 (0.38-0.51)**	**0.58 (0.50-1.04)**	**0.40 (0.35-0.56)**	**0.60 (0.50-0.91)**	**0.44 (0.43-0.48)**

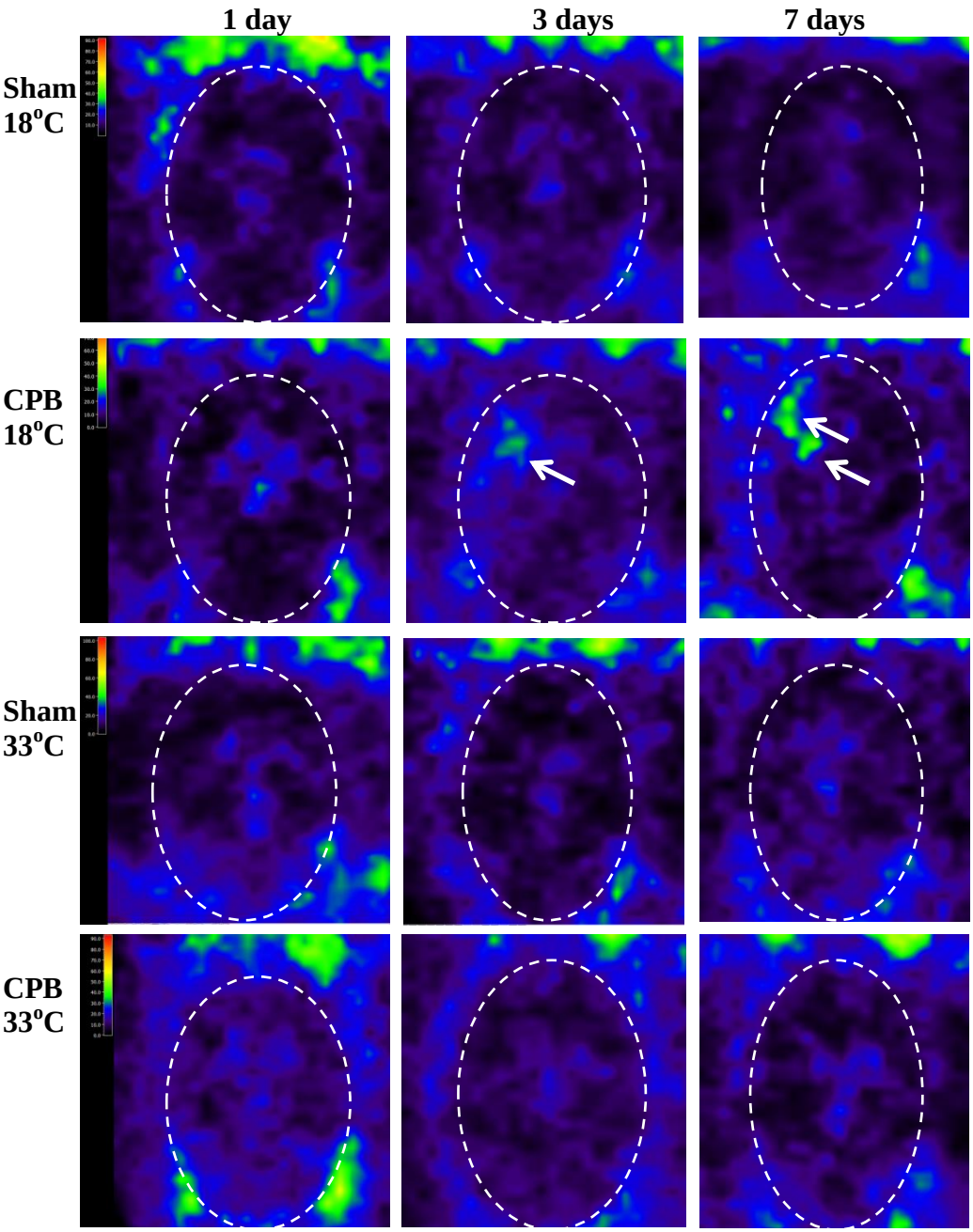


Figure 3. Representative axial PET images of [^{11}C]-PBR28 in CPB and sham operated rats at 18°C and 33°C at 1 day, 3 days and 7 days after procedures. Cerebral circumference is shown as a white dashed line. Arrows point out the hot-spots of PBR28 activity, other than the uptake in the cerebral ventricle system.

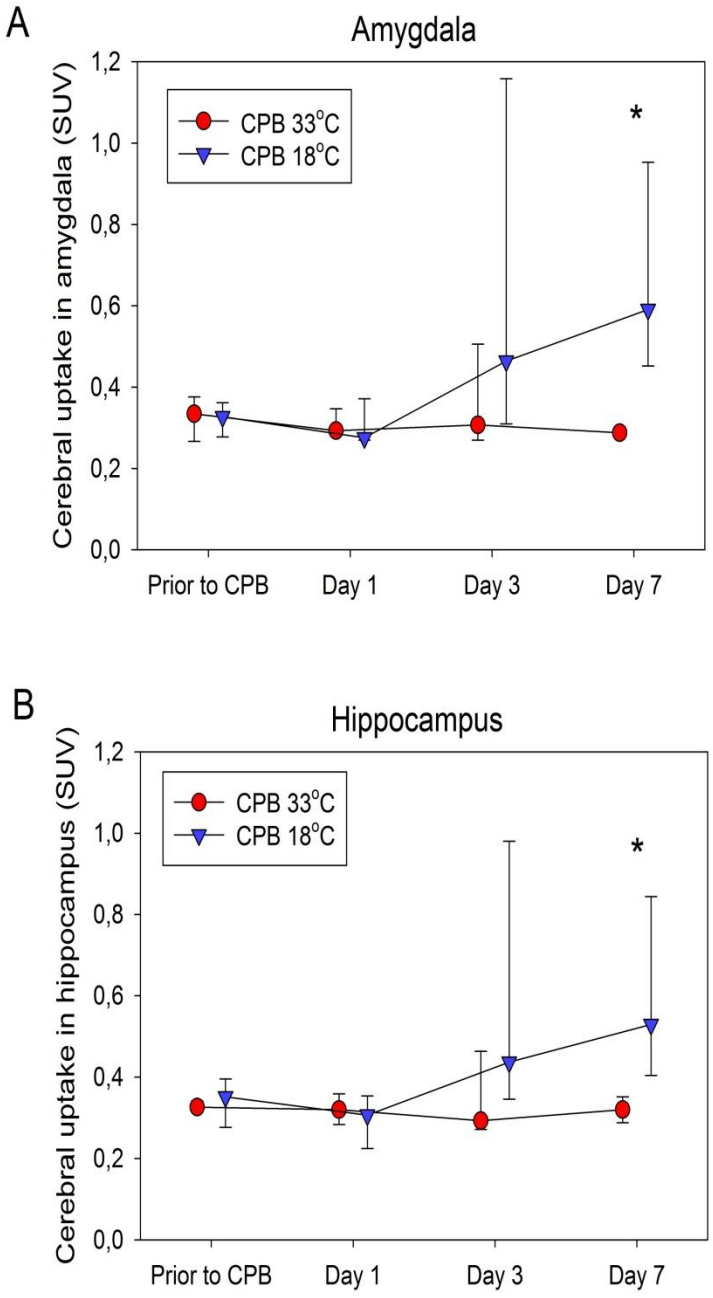


Figure 4. Standard uptake values of [^{11}C]-PBR28 in amygdala (panel A) and hippocampus (panel B) regions of CPB operated rats at 18°C and 33°C prior to and at day 1, 3 and 7 after CPB procedures. Significance is indicated with * for $p<0.05$.

Cold shock proteins

Rat brains were dissected immediately after decapitation and extirpation from the skull. No signs of necrotic tissue or cerebral infarction were present. We analysed TrkB receptor and RBM3 protein levels at day 1 and day 7 in cortex and hippocampus. TrkB in cortex of CPB groups appeared relatively lower than in sham both at 33^{o}C and 18^{o}C at day 1, but not significant. However, TrkB and RBM3 expression in cortex was increased in 33^{o}C CPB at day 1, compared to 18^{o}C CPB (Figure 5A,B; p=0.007 and p=0.019, respectively). At day 7, TrkB and RBM3 in cortex were similar in all groups. In hippocampus, TrkB protein expression levels at day 1 was similar for all groups (Figure 5C). However, at day 7 hippocampal TrkB was significantly increased in 33^{o}C CPB compared to 33^{o}C sham (p=0.042), and appeared to be higher than 18^{o}C CPB at day 7. A similar pattern was found in hippocampal RBM3 expression, which was similar in all groups on day 1, yet significantly higher in 33^{o}C CPB compared to 33^{o}C sham at day 7 (p=0.027; Figure 5D).

Discussion

In the present study, we demonstrated that CPB under TTM targeted at 18^{o}C was associated with a significantly greater neuroinflammatory response in hippocampus and amygdala compared to CPB under TTM targeted at 33^{o}C at day 7 after CPB. Despite CPB representing a stressful model, as shown by significantly higher levels of glucose and lactate after 55 minutes of cooling in CPB groups compared to sham, there were no signs of neuroinflammation found at any time point in rats that underwent CPB under mild hypothermia. We are the first to report on cold shock protein expression in relation to TTM in a cardiopulmonary bypass model. Interestingly, we found induction of TrkB receptor and RBM3 in tissue of CPB rats under mild hypothermia, but an absence of induction in the deeply cooled group. Together, these data demonstrate that mild hypothermia might have advantages in terms of neuroprotection over deep hypothermia in CPB-assisted surgery, associated with increased expression of cold shock proteins in cortex and hippocampus.

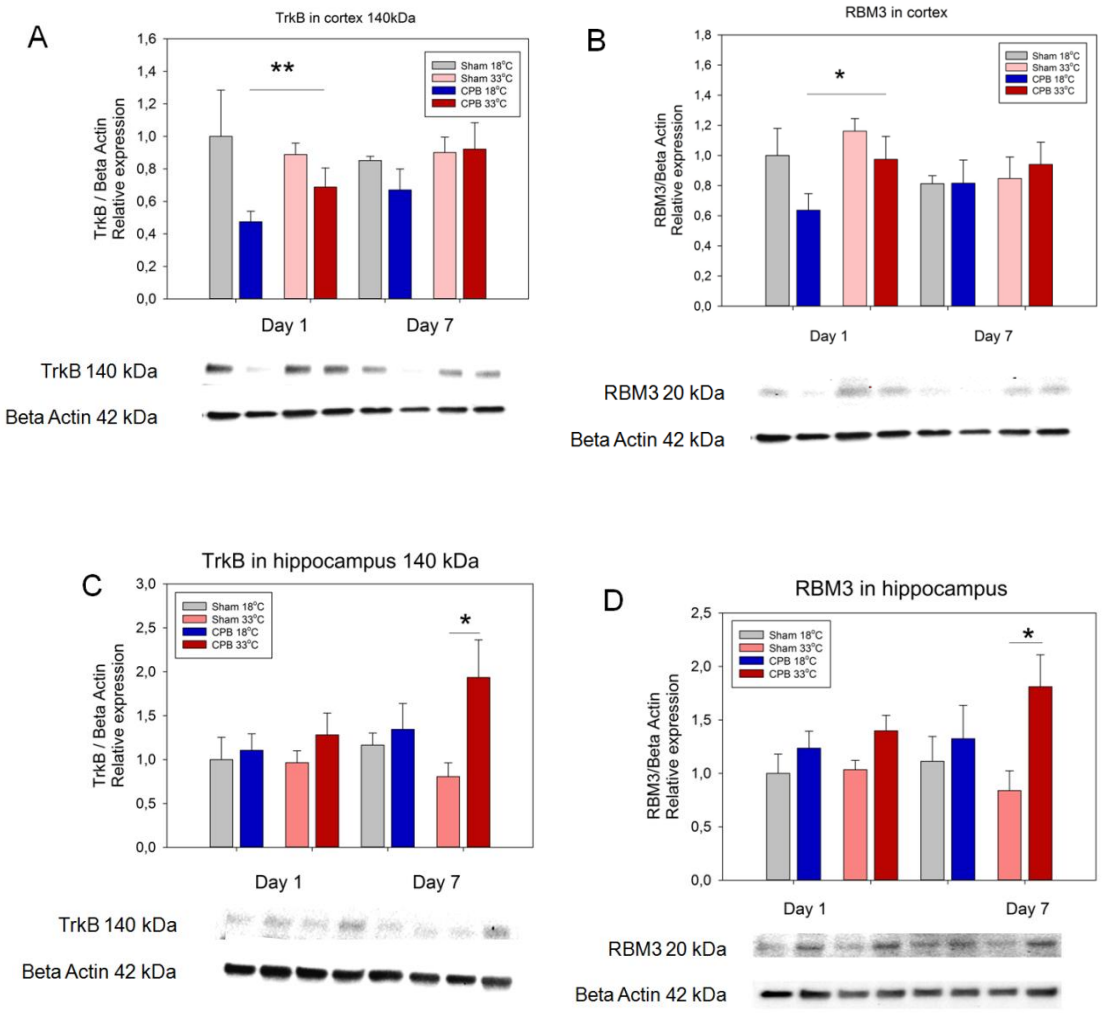


Figure 5. Protein analysis on cold shock proteins in CPB and sham operated rats at 18°C (blue and grey colored bars) and 33°C (red and pink colored bars) at 1 day and 7 days after procedures. TrkB receptor and RBM3 levels in cortex (panel A and B) and in hippocampus regions (panel C and D, respectively). Significance is indicated with */** for $p<0.05$ and $p<0.01$, respectively.

Neuroinflammation in cardiopulmonary bypass

In the current study we applied a CPB model which induces systemic inflammation and renal injury (Samarska et al., 2013; Bouma et al., 2013). The causative factors of neurological injury in human CPB constitute a combination of ischemia and reperfusion (I/R) injury of the brain, resulting from atherosclerosis and cerebral microembolism, cerebral hypoperfusion, arrhythmia, and a substantial systemic inflammatory response (Newman et al., 2006). Neuroinflammation after cardiopulmonary bypass surgery may put a high burden on both systemic and neurological outcome. Even under hypothermic conditions, an innate immune response is triggered. Neonates undergoing CPB-assisted aortic arch reconstruction, who had a short period (30 minutes) of cerebral ischemia during deep hypothermia, showed an immediate inflammatory response in the cerebral circulation, which was significantly greater than in continuously perfused brains (Algra et al., 2013). Neutrophils and monocytes acquired an activated phenotype upon departing the cerebral circulation and increased in number. A study investigating hypothermia at 15^{o}C in CPB found hypothermic circulatory arrest (HCA) to provoke excess histological neuronal damage, compared to selective cerebral perfusion and systemic full-flow perfusion (Sasaki et al., 2009). While the authors suggested that deep hypothermia (15^{o}C) on its own might induce neurological injury, the statement lacks support of adequate temperature control groups. Our findings, however, corroborate this presumption by showing that deep hypothermia is associated with a stronger neuroinflammatory response than mild hypothermia. To extend this statement to an anti-inflammatory effect of mild hypothermia, additional experiments with both moderate hypothermia (28-32^{o}C) and normothermia (35-37^{o}C) would be necessary.

Neuroprotective cold shock

The neuroprotective effect of TTM is influencing many different pathways, e.g. by reducing glutamate release and oxidative stress, and inhibiting release of pro-inflammatory cytokines. In I/R injury models, TTM counteracts mitochondrial induced apoptosis, neuronal excitotoxicity, and neuroinflammation (Talma et al., 2016). As the cold shock protein RBM3 has a post-transcriptional regulatory effect on mRNA stability and translation, it might be the driving force in many of the aforementioned neuroprotective pathways. RBM3 induced by 5'-AMP

induced cooling in mice displayed neuroprotective properties in the 5xFAD model of Alzheimer's disease and in prion infected mice, through the capacity to regenerate synapses (Peretti et al., 2015). Interestingly, we found the highest levels of RBM3 protein in CPB 33°C both in cortex and hippocampus. Concomitant neuroinflammatory responses appeared to play a role in the time of induction of RBM3, as protein levels were significantly elevated at day 1 in cortex, whereas the highest increase in hippocampus tissue was seen at day 7. Beneficial effects of mild hypothermia at 33°C are currently being investigated more thoroughly. Indeed, mild hypothermia may be superior to normothermia with respect to neuroprotection, as suggested by a reduction in brain cytokines IL-1β and TNF-α in a murine stroke model using pharmacologically induced mild hypothermia (33°C) (by applying neurotensin receptor 1 agonist HPI-201) compared to normothermic controls (Lee et al., 2016). Additionally, RBM3 is considered to become upregulated through increased levels of BDNF. Ketamine treatment is known to induce the production of BDNF (Haile et al., 2014). However, ketamine is seldom used in anaesthetic procedures in humans. Hence, more research is necessary to evaluate cold shock response under different anaesthetic agents.

[^{11}C]-PBR28 PET-scans for neuroinflammation

To our knowledge, this is the first study analyzing the effect of hypothermia on neuroinflammation by applying microglial targeted PET scans. Previous studies have shown that the SUV of [^{11}C]-PBR28 shows a strong correlation with distribution volume, together with good reproducibility in mice and rats (Tóth et al., 2015). Utilization of [^{11}C]-PBR28 PET-scans was chosen above immunohistochemistry to enable follow-up of neuroinflammatory processes in the same animal, and because of its translational properties for future clinical studies. As expected, no animals had signs of neuroinflammation prior to the procedure, nor did we observe a correlation between pre- and post-procedural neuroinflammation. However, we found strong correlations between SUV at day 3 and day 7 in amygdala and hippocampal regions, indicative of ongoing neuroinflammatory processes rather than de novo SUV increases after 7 days.

Hypothermic models in rodents

There are several methods and challenges for inducing and monitoring hypothermia in rodents (Klahr et al., 2016). We monitored temperature continuously with a wired thermocouple device and a flexible rectal probe inserted 5cm, to measure core body temperature. Hypothermia was induced at a standardized constant rate either by the CPB circuit or through external temperature application as was performed earlier (Dugbartey et al., 2015). Although we maintained a rewarming rate of up to 2^{o}C per 5 minutes, we prevented post-procedural hyperthermia by actively rewarming to a core body temperature of 35^{o}C. Rewarming was performed by heating the CPB circuit with warm water, or by warm air for sham, in addition to a heating pad. From 35^{o}C onward, only the heating pad was used for rewarming, whereas CPB and warm air were ceased. Hence, core body temperatures above 37.5^{o}C were not registered during weaning. After weaning, animals were placed in a warmed cage for recovery for at least 4 hours. Moreover, last registered temperatures were similar in all treatment groups.

Acknowledgements

This study was partially funded by a grant received from the National Institute of Academic Anaesthesia, UK. RBM3 and TrkB antibodies, together with Western blot consumables were kindly provided by prof. G.R. Mallucci, department of Clinical Neurosciences, Cambridge, UK.